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(54) Title: BIFIDOBACTERIA CAPABLE OF PREVENTING DIARRHEA

(57) Abstract: The present invention pertains to the use of microorganisms belonging to the genus Bifidobacterium for preparing a carrier for the treatment or prophylaxis of diarrhea. The invention also relates to food or pharmaceutical compositions containing such microorganisms.

Bifidobacteria capable of preventing diarrhea

The present invention pertains to the use of non-pathogenic microorganisms of the genus Bifidobacterium for preparing a carrier for the treatment or prophylaxis of diarrhea brought about by rotaviruses, and to food or pharmaceutical compositions containing such micro-organisms.

Organisms that produce lactic acid as a major metabolic component have been known for a long time. These bacteria may be found in milk or in milk processing factories, respectively, living or decaying plants but also in the intestine of man and animals. These microorganisms, summarized under the term "lactic acid bacteria", represent a rather inhomogeneous group and comprise e.g. the genera Lactococcus, Lactobacillus, Streptococcus, Bifidobacterium, Pediococcus etc..

Lactic acid bacteria have been utilized as fermenting agents for the preservations of food taking benefit of a low pH and the action of fermentation products generated during the fermentative activity thereof to inhibit the growth of spoilage bacteria. In addition, lactic acid bacteria have also been used for preparing from milk a variety of different foodstuff such as cheese, yogurt and other fermented dairy products.

Quite recently, lactic acid bacteria have attracted a great deal of attention in that some strains have been found to exhibit valuable properties to man and animals upon ingestion. In particular, specific strains of Lactobacillus or Bifidobacterium have been found to be able to colonize the intestinal mucosa and to assist in the maintenance of the well-being of man and animal.

In this respect, EP 0 768 375 discloses specific strains of the genus Bifidobacterium, that are capable to become implanted in the intestinal flora and may adhere to intestinal cells. These

Bifidobacteria are reported to assist in immunomodulation, being capable to competitively exclude adhesion of pathogenic bacteria to intestinal cells, thus assisting in the maintenance of the individual's health.

During the last few years research has also focused on the potential use of lactic acid bacteria as probiotic agents. Probiotics are considered to be viable microbial preparations which promote the individual's health by preserving the natural microflora in the intestine. A microbial preparation may be commonly accepted as a probiotic in case the effectual microbes thereof and their mode of action are known. Probiotics are deemed to attach to the intestine's mucosa, colonize the intestinal tract and likewise prevent attachment of harmful microorganisms thereon. A crucial prerequisite for their action resides in that they have to reach the gut's mucosa in a proper and viable form and do not get destroyed in the upper part of the gastrointestinal tract, especially by the influence of the low pH prevailing in the stomach.

In this respect, WO 97/00078 discloses a specific strain, termed Lactobacillus GG (ATCC 53103), as such a probiotic. The microorganism is particularly employed in a method of preventing or treating food induced hypersensitivity reactions in that it is administered to a recipient together with a food material that has been subjected to a hydrolysis treatment with pepsin and/or trypsin. The Lactobacillus strain selected is described as exhibiting adhesive and colonizing properties and showing a protease enzyme system, so that the protein material contained in the foodstuff to be administered is further hydrolysed by means of proteases secreted by the specific Lactobacillus strain. The method discussed in this document shall eventually result in the uptake of protein material by the gut that does not show a substantial amount of allergenic material anymore.

Further, in EP 0 577 903 reference is made to the use of such lactic acid bacteria having the ability of replacing Heliobacter pylori, the acknowledged cause for the development of ulcer, in the preparation of a support intended for the therapeutic or prophylactic treatment of an ulcer associated with the action of Heliobacter pylori.

In view of the valuable properties particular strains of lactic acid bacteria may exhibit there is a desire in the art to find additional properties of bacterial strains beneficial to the well being of man and/or animal.

Consequently, the problem underlying the present invention is to provide additional lactic acid bacteria that may exert beneficial activities to living beings upon ingestion.

In the course of the studies leading to the invention it was now surprisingly found that microorganisms of the genus Bifidobacterium show properties not yet recognized in the art. In effect, the present invention provides for the use of microorganisms belonging to the genus Bifidobacterium and being capable to essentially prevent infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.

The Bifidobacteria to be used are preferably selected from the group consisting of Bifidobacterium adolescentis or Bifidobacterium longum, preferably Bifidobacterium adolescentis, and is more preferably Bifidobacterium CNCM I-2168.

The microorganisms may be used for the preparation of a variety of ingestable carriers, such as e.g. milk, yogurt, curd, fermented milks, milk based fermented products, fermented cereal based products, milk based powders, infant formulae or pet food and may be included in the respective carrier in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g. For the purpose of the present invention the abbreviation cfu shall designate a "colony forming unit" that is defined as number of bacterial cells as revealed by microbiological counts on agar plates.

The present invention also provides for a food or a pharmaceutical composition containing at least one of the Bifidobacterium strains capable to essentially prevent infection of intestinal cells by rotaviruses.

For preparing a food composition according to the present invention at least one of the Bifidobacterium strains used according to the present invention is incorporated in a suitable

support, in an amount of from about 10^5 cfu / g to about 10^{11} cfu / g, preferably from about 10^6 cfu / g to about 10^{10} cfu / g, more preferably from about 10^7 cfu / g to about 10^9 cfu / g.

In case of a pharmaceutical preparation the product may be prepared in form of tablets, liquid bacterial suspensions, dried oral supplements, wet oral supplements, dry tube feeding or a wet tube feeding etc., with the amount of Bifidobacterium strains to be incorporated therein being in the range of up to 10^{12} cfu / g, preferably from about 10^7 cfu / g to about 10^{11} cfu / g, more preferably from about 10^7 cfu / g to about 10^{10} cfu / g.

The microorganisms may further be formulated in the carrier so as to obtain a desired release pattern, such as encapsulation etc. Based upon the desired objective the person skilled in the art will select the appropriate excipients and/or additives.

The activity of the microorganisms in the individual's intestine is of course dose dependent. That is, the more the microorganisms are incorporated by means of ingesting the above food material or the pharmaceutical composition, respectively, the higher the protective and/or curing activity thereof. Since the used microorganisms are not detrimental to mankind and animals and have eventually been isolated from a natural surrounding, namely baby feces, a high amount thereof may be incorporated so that essentially a high proportion of the individual's intestine will be colonized by the microorganisms.

In the figures,

Fig. 1 shows a scheme illustrating the cell culture screening for assessing rotaviral protective properties of bacterial strains.

During the extensive studies leading to the present invention the inventors have investigated different bacterial strains isolated from baby feces or obtained from the American Tissue and Cell Collection (ATCC 15704). The different strains were examined for their capability to prevent infection of intestinal cells with rotaviruses that are known to cause diarrhea.

Several bacterial genera comprising Bifidobacterium, Lactococcus, Streptococcus were screened for their rotavirus inhibitory properties. The tests for the inhibitory property were essentially performed with three rotavirus serotypes representing the major etiological agents of human viral diarrhea (serotypes G1, G3 and G4).

The various lactic acid bacteria were grown in a suitable medium, such as MRS, Hugo-Jago or M17 medium at temperatures of from about 30 to 40°C corresponding to their optimal growth temperature. After reaching stationary growth the bacteria were collected by centrifugation and re-suspended in physiological NaCl solution. Between the different tests the bacterial cells were stored frozen (-20°C).

The various rotavirus stocks were prepared by infection of confluent cell monolayers. The rotaviruses were incubated before infection. The cells were infected with 20 tissue culture infectious doses.

For assessing anti-rotaviral properties two different protocols were applied. According to one protocol the various bacterial strains were examined for their direct interaction with the rotavirus strain while in the second protocol the bacteria were screened for those strains that interact with cellular rotavirus receptors.

The first protocol involved contacting the respective bacterial suspension each with a different rotavirus strain and incubating in suitable media. Subsequently, the virus-bacteria mixture was applied to a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 (intestinal epithelial cell line) and incubation was continued. Virus replication was then assayed.

The second protocol involved incubating the respective bacterial suspension first together with a monolayer of cells of the human undifferentiated colon adenoma cells HT-29 and adding the virus subsequently. After continued incubation virus replication was assayed.

Rotavirus replication may easily be assessed by histo-immunological staining of rotavirus proteins in infected cells.

A rotavirus inhibitory effect was attributed to a given bacterium when the number of infected cells was reduced by 90% in the cell culture inoculated with rotavirus plus the indicated bacteria in comparison with cells inoculated only with rotavirus.

Out of a total of 260 different bacterial strains primarily isolated merely 4 could be shown to essentially inhibit rotaviral replication. The different bacteria were ascertained to belong to the genus Bifidobacterium subspecies adolescentis or longum. One strain belonging to the species Bifidobacterium adolescentis, which has been termed Bad4, has been deposited in accordance with the Budapest Treaty and has received the deposit number CNCM I-2168. This strain proved to be extremely effective in preventing infection of human cells by rotaviruses.

The present invention will now be described by way of example.

Media and solutions:

MRS (Difco),

Hugo-Jago (Tryptone Difco 30 g/l, Yeast Extract Difco 10 g/l, Lactose Difco 5 g/l, KH_2PO_4 5 g/l, Beef Extract Difco 2 g/l, agar Difco 2 g/l)

M17 (Difco)

M199 (Seromed)

Ringer solution (Oxoid)

PBS (NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l))

Tryptose phosphate broth (Flow)

Trypsin-EDTA solution (Seromed)

Human rotavirus Wa (G1 serotype) and simian rotavirus SA-11 (G3 serotype) were obtained from P.A. Offit, Children's Hospital of Philadelphia, U.S.A. The DS-1xRRV reassortant

virus was obtained from A. Kapikian, NIH Bethesda, U.S.A. The serotype 4 human rotavirus Hochi was obtained from P. Bachmann, University of Munich, Germany.

Example 1

Isolation of lactic acid bacteria from baby feces

Fresh feces were harvested from diapers of 16 healthy babies 15 to 27 days old. 1 g of fresh feces was placed under anaerobic conditions for transportation to the laboratory and microbiological analyses were run within 2 hours from sampling by serial dilutions in Ringer solution and plating on selective media. MRS agar plus antibiotics (phosphomycine 80 µg/ml, sulfamethoxazole 93 µg/ml, trimethoprime 5µg/ml) incubated at 37°C for 48 hours was used to isolate lactic acid bacteria. Colonies were randomly picked up and purified. Physiological and genetic characterisation was performed on the isolates. In the tests another strain obtained from ATCC (ATCC 15704) was also used, which corresponds to the preferred strain Bad4 to be used according to the present invention.

Example 2

Testing of strains in cell culture for anti-rotaviral activity

Several bacterial genera comprising Bifidobacterium, Lactococcus and Streptococcus were selected and tested for members which showed anti-rotaviral activity in the cell culture inhibition test (see below 1st and 2nd protocol). The genus Lactococcus was represented by a single species (Lc. lactis) consisting of two subspecies (Lc. lactis supsp. lactis and cremoris). A total of 30 strains were tested. The Streptococcus genus was represented by a single species (S. thermophilus) with 45 strains. The Leuconostoc and Propionibacterium genus were only represented by a single species (6 strains), while the Enterococcus and Staphylococcus genus was represented by two species each and a total of 17 strains.

In total, 260 bacterial strains were tested for rotavirus inhibitory activity.

1st protocol:

30 µl of the respective bacterial suspension (containing on average $3x10^6$ bacteria) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) to which were added 100 µl of virus in supplemented M199 medium. The virus–bacteria mixture thus obtained was incubated for 1 hour at 4°C and for 1 hour at 37°C. Separately, cells of the human undifferentiated colon adenoma cells HT-29 growing as a confluent monolayer in 96-well microtiter plates (in M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) 1 : 4 diluted with PBS) were washed three times with phosphate-buffered saline (PBS ; pH 7.2). The virus-bacteria mixture processed as indicated above was transferred to the cells and the microtiter plates were incubated for 18 h in a GO_2 incubator (Heraeus). Virus replication was assayed as described below.

2nd protocol:

30 µl of the bacterial suspension (supra) were mixed with 70 µl M199 medium supplemented with 10% tryptose phosphate broth (Flow) and 5% trypsin-EDTA solution (Seromed) and applied directly on HT-29 cells grown and pretreated as described in the 1st protocol in the microtiter plates. After one hour incubation at 37°C 100 µl of virus in supplemented M199 medium were added to the cells in the microtiter plates. The incubation was continued for 18 h in a CO_2 incubator (Heraeus). Virus replication was assayed as described below.

The rotavirus replication was assessed by histo-immunological staining of rotavirus proteins in infected cells as described hereafter.

One day after infection, the cell culture medium was removed from the microtiter plates and the cells were fixed with absolute ethanol for 10 min. Ethanol was discarded, and the plates were washed three times in PBS buffer. Then 50 µl of an anti-rotavirus serum (mainly directed against VP6 protein), produced in rabbits (obtained from the ISREC University of Lausanne) and diluted 1 :2000 in PBS was added to each well and incubated for 1 h at 37°C with a cover slip to prevent desiccation of the wells. The anti-serum was discarded afterwards

and the plates were washed three times with PBS. Then 50 µl of anti-rabbit immunoglobulin G (IgG) antiserum produced in goats and coupled to peroxidase (GAR-IgG-PO; Nordic) were added at a dilution of 1 : 500 in PBS to each well and the plates were incubated for 1 hour at 37 °C. The serum was discarded and the plates were again washed three times with PBS. Then 100 µl of the following substrate mixture was added to each well : 10 ml of 0.05 M Tris-hydrochloride (pH 7.8), 1 ml of H_2O_2 (30% suprapur, diluted 1 :600 in H_2O ; Merck) and 200 µl of 3-amino-9-ethylcarbazole (0.1 g/10 ml of ethanol stored in 200 µl aliquots at – 80 °C ; A-5754 ; Sigma). The plates were incubated for at least 30 min at room temperature. The substrate was discarded and the wells were filled with 200 µl of H_2O to stop the reaction. Infected cell foci were counted with an inverted microscope (Diavert ; Leitz).

Only very few bacterial strains interacted with rotaviruses. Merely 4 out of the 260 bacterial cells primarily selected inhibited rotavirus replication in at least one protocol. Bifidobacterium adolescentis CNCM I-2168 (Bad4) showed an extremely high activity against Serotype 1 Rotavirus, Serotype 3 rotavirus SA-11 and Serotype 4 rotavirus Hochi.

Bad4 is gram positive and catalase negative, it does not produce CO_2 during fermentation and produces just L (+) lactic acid according to methods disclosed in the "Genera of lactic acid bacteria", Ed. B.J.B. Wood and W.H. Holzapfel, Blackie A&P.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 3, line 16.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
Collection Nationale de Cultures de Microorganismes
Institut Pasteur

Address of depositary institution *(including postal code and country)*
25, Rue du Docteur Roux
F-75724 Paris Cedex 15

Date of deposit	Accession Number
15/03/1999	NCC 251 - I-2168

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Mrs. H. Fransz	Authorized officer

Form PCT/RO/134 (July1998)

Claims

1. Use of a lactic acid bacterium belonging to the genus Bifidobacterium capable of preventing infection of intestinal cells by rotaviruses for the preparation of a carrier for the treatment or prophylaxis of diarrhea.

2. The use according to claim 1, wherein the Bifidobacterium is selected from the group consisting of Bifidobacterium longum or Bifidobacterium adolescentis.

3. The use according to claim 1, wherein the Bifidobacterium is Bifidobacterium CNCM I-2168.

4. The use according to any of the preceding claims, wherein the Bifidobacterium is contained in an ingestable carrier.

5. The use according to claim 5, wherein the Bifidobacterium is contained in the carrier in an amount from about 10^5 cfu / g to about 10^{12} cfu / g carrier.

6. The use according to any of the claims 4 or 5, wherein the carrier is a food composition selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae or pet food.

7. Food or pharmaceutical composition containing at least one Bifidobacterium strain capable of preventing infection of intestinal cells by rotaviruses.

8. The composition according to claim 7, which is selected from milk, yogurt, curd, cheese, fermented milks, milk based fermented products, ice-creams, fermented cereal based products, milk based powders, infant formulae, pet food, tablets, liquid bacterial suspensions, dried oral supplement, wet oral supplement, dry tube feeding or wet tube feeding.

9. The composition according to claim 7, which is in form of a tablet, liquid bacterial suspension, dried oral supplement, wet oral supplement, dry tube feeding or a wet tube feeding.

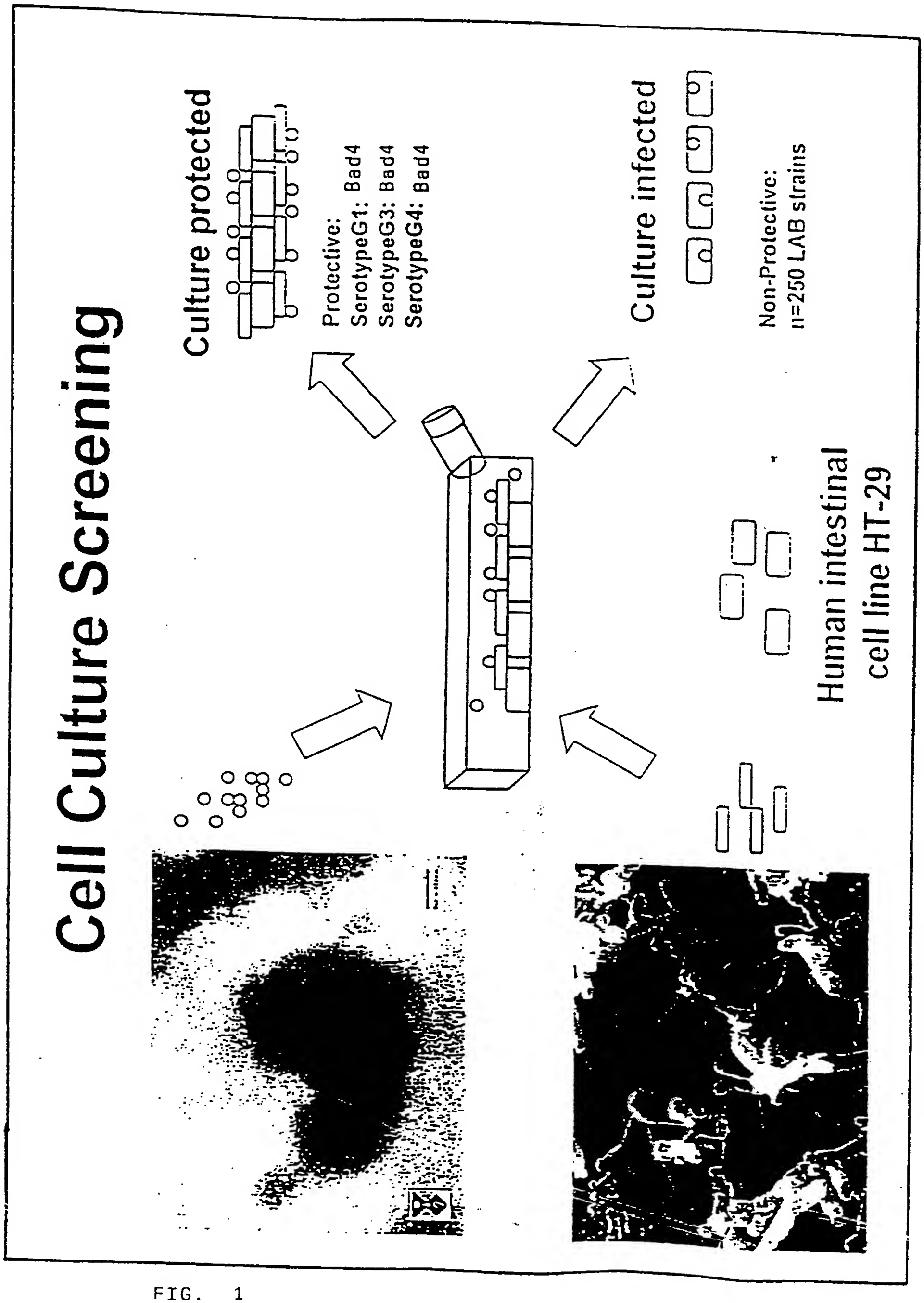
Cell Culture Screening
Culture protected
Protective:
SerotypeG1: Bad4
SerotypeG3: Bad4
SerotypeG4: Bad4
Culture infected
Non-Protective:
n=250 LAB strains
Human intestinal
cell line HT-29

FIG. 1